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Intracellular parasitism and molecular determinants of *Legionella* virulence

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Summary Bacteria of the genus *Legionella* are intracellular parasites and major human pathogens. They bind to surface receptors, penetrate eukaryotic cells and initiate complex disorders during phagocytosis. These disorders include inhibition of oxidative burst, a decrease in phagosome acidification, the blocking of phagosome maturation and changes in organelle trafficking. As a result, the microorganisms prevent the bactericidal activity of the phagocyte and transform the phagosome into a niche for their replication. Biological, biochemical and molecular-genetic approaches have been used to identify a panel of bacterial products that may be involved in *Legionella* virulence. They include cytotoxins, several enzymes and a set of genes thought to encode proteins of the export machinery. However, despite distinct progress in research, the molecular mechanisms underlying intracellular parasitism in *Legionella* are unclear.

Key words *Legionella pneumophila* · Legionellosis · Pathogenesis · Phagocytosis ·

Legionella and legionellosis

The history of *Legionella* began in 1976, when a large outbreak of severe pneumonic illness occurred among visitors to the Legionnaires' convention in Philadelphia, USA. In 1977 the causal microorganism was isolated and named *Legionella pneumophila*. In the following years, additional species were identified and the number of species is now more than 50 [47b]. Most of these *Legionella* species are reported to be pathogenic for humans.

Legionellae are ubiquitous microorganisms. They have been isolated from water and soil specimens with a broad spectrum of physico-chemical characteristics. The widespread distribution of the bacteria can be attributed not only to their high adaptive potential, but also to their ability to grow in association with other bacteria, algae and, most importantly, protozoa [2, 61, 64]. As a result, legionellae colonize not only natural aquatic environments, but also human-made water reservoirs (e.g. cooling towers, air-conditioning systems, drinking and hot water distribution systems). In such systems, the concentration of virulent bacteria may reach high levels and represent a real danger for exposed individuals.

The predominant clinical manifestation of legionellosis is severe pneumonia (Legionnaires' disease). There is also another form of the disease, an acute self-limiting non-pneumonic type called Pontiac fever. The disease begins with the inhalation of a sufficient dose of virulent bacteria. Microorganisms in small aerosol droplets get to alveoli and are ingested by lung macrophages or invade lung epithelial cells. In these cells, microorganisms multiply, kill phagocytes and invade surrounding cells while the infection progresses.

Early pathological studies indicated the critical importance of intracellular multiplication of the bacterium for the development of full Legionnaires' disease. This was confirmed experimentally in later investigations, in which mutated strains of *L. pneumophila*, defective in intracellular multiplication, were found to be severely impaired in the ability to cause experimental infection in animals. Thus intracellular multiplication is a critical step in the pathogenesis of legionellosis.

Interaction of *Legionella* with phagocytic cells

The interaction of virulent legionellae with phagocytic cells can be arbitrarily divided into several steps: binding of microorganisms to receptors on the surface of eukaryotic cells, penetration of microorganisms into phagocytes, escape from bactericidal attack, formation of replicative vacuole; intracellular multiplication, and killing of the host cell.

The first step in host-parasite interactions is the binding of the microorganism to receptors on the surface of a target cell. Initial experiments demonstrated that receptors for the C3 component of complement could participate in the binding

process [42]. In their next study, the authors showed that the bacteria fixed C3 and that this fixation was accomplished selectively by major outer membrane proteins (MOMP, porin, P29). Thus, the C3 component is an intermediary between MOMP and the corresponding eukaryotic receptor. The binding of C3 may be targeted to specific domains of the P29 molecule because cyanogen bromide-generated fragments bound complement components differentially. The ability of *L. pneumophila* porin to fix C3 and to mediate phagocytosis was further characterized by MOMP reconstituted into liposomes. The MOMP-liposomes produced not only readily fixed the C3 component, but they then adhered to and were internalized by human monocytes [6].

Thus, the porin of *L. pneumophila* may direct phagocytosis via a specific pathway (using C3 components of complement and the corresponding phagocyte receptors). However, it is not clear if this internalization route leads to the killing of bacteria or their proliferation (i.e. is it advantageous for phagocytes or for legionellae?). MOMP is a species-specific protein and is found only in the representatives of a single species, *L. pneumophila*. It is unclear which of the bacterial components of other pathogenic *Legionella* species are involved in the ligation of C3 receptors. It is also unknown whether P29 binds a receptor on the surface of protozoa and mediates phagocytosis in such hosts.

Common sense suggests that internalization of *Legionella* cells could be achieved by other complement-independent pathways as well. Possible candidates for complement-independent ligands include bacterial flagella and pili.

The flagella of *Legionella* consist of protein subunits with a molecular weight of 47 kDa and have been detected in many representatives of the genus. The *fliI* gene, coding for flagellin, has been cloned and sequenced. Insertion mutation technology has been used to generate an unflagellated strain of *L. pneumophila*. It was found that the mutated and wild-type strains multiplied equally well in the macrophage-like cell line U937 [39]. This finding raised questions about the role of flagella in intracellular parasitism by *L. pneumophila*. However, the study in question does not rule out the possibility of flagella being involved in the pathogenesis of *Legionella* infection. Indeed, the defined mutant was tested only on macrophage-like cells. It is possible that other cell types (alveolar macrophages, epithelial cells or protozoa) have special receptors and microorganisms can use flagella as ligands to bind such receptors.

Clear demonstration has been obtained of the importance of pili in host cell-*Legionella* interactions. A gene encoding the protein components of pili has been identified and cloned. It had high homology with type IV pilin genes of *Neisseria* and *Pseudomonas aeruginosa* and was named $pilE_L$ [58]. Mutation of the $pilE_L$ gene resulted in the disappearance of long pili from the surface of *L. pneumophila*. Simultaneously, the strain mutated in this way showed a decrease in attachment to certain cell types (human epithelial cells and amoebae) of about 50%. The rate of intracellular replication of mutated strains, however, did not differ drastically from that of the wild-type legionellae. Thus, $pilE_L$ is clearly important in the first steps of *Legionella* interaction with target eukaryotic cells. However, Southern blot analysis of the distribution of the $pilE_L$ locus among various representatives of the genus *Legionella* demonstrated that certain pathogenic species (e.g. *L. dumoffii*, *L. gormanii*, *L. micdadei*) do not possess such a gene. These results suggest there may be other types of surface structure necessary for the attachment of bacteria to phagocytic cells.

Binding to certain eukaryotic receptors may be the first mechanism utilized by *Legionella* to direct phagocytosis to the advantage of the bacterium and to prevent bactericidal attack. This idea is purely hypothetical due mostly to the lack of information about specific eukaryotic receptors for this bacterium. Some progress has been made in determining the cell receptor for *Legionella* using a protozoan model. It has been reported that a particular galactose lectin mediates attachment of the bacterium to *Hartmanella vermiformis*. This lectin receptor is similar to eukaryotic integrin-like receptors and could presumably initiate cellular signaling processes regulating the course of phagocytosis [1, 62].

Attachment of bacteria to the surface structures of eukaryotic cells may lead to the internalization of microorganisms. Penetration of *Legionella* into phagocytes has been shown to be accomplished by two morphologically different mechanisms: phagocytosis by “zipper” and “coiling” mechanisms. In the zipper mechanism, the cytoplasmic membrane surrounds the microorganism which is then translocated into the cell inside the phagocytic vacuole. This type of phagocytosis has also been described for *Mycobacterium*, *Listeria* and *Yersinia* [26]. Coiling phagocytosis has been observed only during the invasion of eukaryotic cells by strains of *L. pneumophila* serogroup 1. In this process, a long phagocyte pseudopodium coils around the bacterium as it is being internalized. At the end of this process, the *L. pneumophila* cell remains at the center of a large spiral. The external portion of the coil then disintegrates and the organism remains in a conventional membrane-bound phagosome. This type of phagocytosis has also been shown for other microorganisms [48].

The internalization of *Legionella* is one of the least investigated phases of the bacteria-host cell interaction. Nothing is known about molecular cross-talk between the phagocyte and the microorganisms, or whether the mechanisms of translocation utilized by *L. pneumophila* are unique and different from processes occurring during the internalization of other intracellular parasites.

After translocation into eukaryotic cell, legionellae are able to escape from bactericidal attack by phagocytes. They achieve this by inhibiting phagosome-lysosome fusion, decreasing phagosome acidification and preventing oxidative burst generation [33, 34, 44]. As the molecular mechanisms underlying the observed changes may relate to eukaryotic

membrane-driven events, attempts have been made to study changes in the membrane of the phagocytic vacuole.

Studies on the composition of the vacuole membrane during development and the maturation of the phagosome containing virulent *L. pneumophila* have demonstrated complex quantitative and qualitative rearrangements of its structure different from those observed during phagocytosis of avirulent bacteria. The internalization of virulent *L. pneumophila* results in the exclusion of several plasma membrane proteins from the phagosome, along with major histocompatibility complex I and II molecules, and the failure to accumulate certain markers, such as transferrin receptor, CD63, LAMP-1, LAMP-2, and cathepsin D [23]. Thus, the maturation of the *L. pneumophila*-containing vacuole was blocked. However, the molecular mechanisms behind these changes and their role in both *Legionella* and phagocyte biology are unknown.

These malfunctions in phagocytosis have been shown to be accompanied by organelle recruitment. Thus, following internalization, phagosomes containing *Legionella* sequentially interact with smooth vesicles, mitochondria and ribosomes. After 15 min phagosomes are surrounded by smooth vesicles. Later, after approximately 1 h, phagosomes are surrounded by mitochondria. And finally, approximately 4 h after internalization, the bacteria-containing phagosomes are covered by ribosomes and ribosome-lined endoplasmic reticulum vesicles. The resulting vacuole is described as a "replicative vacuole" [59].

The next step in *Legionella*-phagocyte interaction is the rapid multiplication of bacteria. Proliferation of microorganisms takes place inside replicative vacuoles. This indicates that legionellae successfully transform the phagosome into a convenient niche for propagation, a necessary requirement of which is a continuous and adequate supply of nutrients. The molecular mechanisms behind this transformation are unknown.

The number of proliferating bacterial cells per target phagocyte may be very high during *Legionella* invasion. Thus, the death of phagocytic cells may be caused by bacterial overload and the resultant lethal damage to eukaryotic organelles. Another documented cause of eukaryotic cell death is the induction of apoptosis [31]. However, programmed cell death has been observed only in infected human monocytes but not, for example, in the protozoan *Acanthamoeba castellanii*. The detailed mechanism of apoptosis induction is unknown. It is also unknown whether legionellae cause the death of host cells by secreting special products with killing activity (e.g. cytotoxin).

Thus there are several prominent features of *Legionella*-phagocyte interactions: (i) phagocytosis is mediated by specific ligands between the surface of bacteria and the corresponding eukaryotic receptors; (ii) phagocytosis may proceed via "zipper" or "coiling" mechanisms; (iii) the maturation of phagosomes containing *L. pneumophila* is blocked; (iv) acidification and fusion of the phagosome to lysosomes are inhibited; (v) the oxygen-dependent killing activity of phagocytes is impaired; (vi) phagosomes retaining *L. pneumophila* sequentially interact with smooth vesicles, mitochondria and ribosomes and are finally surrounded, at "replicative" phagosome phase by rough endoplasmic reticulum; and (vii) human phagocytes infected with virulent *L. pneumophila* are subject to programmed cell death.

It is conceivable that the action of certain microbial products at very early stages in the *Legionella*-phagocyte interaction (e.g. at the phase of bacterial adhesion to cell receptors or at internalization) is sufficient to cause many of the abnormalities in phagocytosis that occur at the later steps. As a consequence of these changes, microorganisms are able to escape the bactericidal action of phagocytes and to multiply in a replicative phagosome.

Products of *Legionella* as molecular determinants of microbial pathogenesis

Biological approach In efforts to find the virulence determinants of legionellae, various approaches have been used. Initially, authors attempted to detect such products in *Legionella* cultures by directly assessing biological activity (lethal activity in animal models or toxic activity in cell culture models). Only at later stages of investigation were potential virulence factors subjected to thorough biochemical and molecular genetic analyses.

The low-molecular weight cytotoxin was one of the first putative virulence factors with toxic activity to be purified from *Legionella* cultures. It was originally identified in supernatants of *L. pneumophila* as being toxic to embryonated hen's eggs and cytotoxic to CHO cell products. The cytotoxin was purified and shown to be a peptide with a molecular weight of ca. 1200 Da. It was later demonstrated that the cytotoxin affected oxidative metabolism in polymorphonuclear phagocytes [28, 37]. At concentrations that did not affect neutrophil viability, the peptide abolished hexosemonophosphate shunt (HMPS) activity and oxygen consumption during phagocytosis, along with the inhibition of iodination and killing of *Escherichia coli* by neutrophils. The effect of cytotoxin on HMPS activity was specific. Thus, hexosemonophosphate shunt activity and O_2^- production stimulated by fMLP, A23187 and latex beads was reduced, whereas the stimulatory action of ConA, PMA and valinomycin was not affected [24].

It is known that fMLP-induced activation of O_2^- production proceeds as a chain of reactions: fMLP receptor $\rightarrow$ G-protein $\rightarrow$ phosphatidylinositol-specific phospholipase C $\rightarrow$ protein kinase C $\rightarrow$ NADPH oxidase. A cytotoxin affects fMLP-induced stimulation of the oxidative burst and does not inhibit activation by PMA (a specific activator of protein kinase C) of O_2^- production, the probable sites of its action preceed protein kinase C activation [24].

In another study using bacterial ultrasonic lysates as a crude preparation, Hedlund and colleagues purified *Legionella* lethal

factor. This product was cytotoxic for macrophages, lethal to AKR/J mice, had a molecular weight of 3400 Da and was present in several species of *Legionella* [32]. During investigations of its effect on neutrophil functions, it was observed that the purified toxin preparation significantly inhibited oxidative burst activity.

Thus, two low-molecular weight toxins have been purified from *Legionella* cultures. The corresponding genes have not been cloned and no data are available about the molecular organization of these genes and regulation of their expression. The precise mechanism of action of neither toxin is known. These toxins may act as "false" messengers in signaling cascades during the regulation of oxygen metabolism, may inactivate enzymes by competing for substrate or may act as "adapter" proteins in eukaryotic protein-protein interactions. Another possibility is that low-molecular weight toxins act as ligands for certain intracellular or surface receptors. However, such ideas are purely hypothetical and require experimental confirmation.

One of the most studied cytotoxic factors identified in *Legionella* is cytolysin, a protein also called Zn-metalloproteinase, a major secretory protein and tissue-destroying protease. In our laboratory, cytolysin was initially detected in *L. pneumophila* liquid cultures by testing on CHO cell monolayers [10]. We observed the rapid rounding and death of CHO cells. Subsequently, a toxic factor was isolated and investigated [11].

Legionella cytolysin is a 38 kDa protein with proteolytic activity against various substrates, including collagen, gelatin, casein, interleukin-2, the CD4 receptor and immunoglobulins [9, 16, 40]. The purified protease also lysed erythrocytes and had toxic effects against HeLa cells and fibroblasts. In animal models, the intratracheal injection of cytolysin produced lesions resembling those of *Legionella* pneumonia and, if introduced in large doses (50–100 micrograms per guinea pig), killed animals, with clinical signs of haemorragic lung edema [Belyi, unpublished].

The *Legionella* protease has also been demonstrated to impair the functioning of phagocytes and natural killer cells [45, 46]. At non-toxic concentrations, it inhibits human neutrophil and monocyte chemotaxis towards various chemoattractants and inhibits the killing in monocytes of another intracellular pathogen, *Listeria monocytogenes*. These data indicate that cytolysin may be involved in impairing diverse phagocyte functions. However, whether protease directly inhibited the functions of phagocytic cells or was involved in the processing and activation of another biologically active molecules is unclear.

We investigated the effect of cytolysin on signaling reactions in eukaryotic cells, in particular eukaryotic protein kinase cascades [7, 15].

Our preliminary experiments indicated that, in the presence of ^{32}P-γATP proteins with molecular weights of approximately 150 kDa and of 55 kDa were phosphorilated, in the cytoplasmic fraction of lung tissue cells. The addition of *Legionella* culture filtrate or purified cytolysin to the reaction system changed the image on the autoradiograph: the 150 and 55 kDa phosphoproteins were no longer detected, but a phosphoprotein with a molecular weight of 45 kDa was present (Fig. 1). We investigated the observed changes in phosphorylation more precisely by partially purifying the components of the protein kinase system for further investigation. We showed that *Legionella* cytolysin specifically cleaved the 55 kDa acceptor protein of the phosphokinase system of lung cells to give a 45 kDa component. The absence of the 150 kDa protein may also be explained by the proteolytic action of cytolysin.

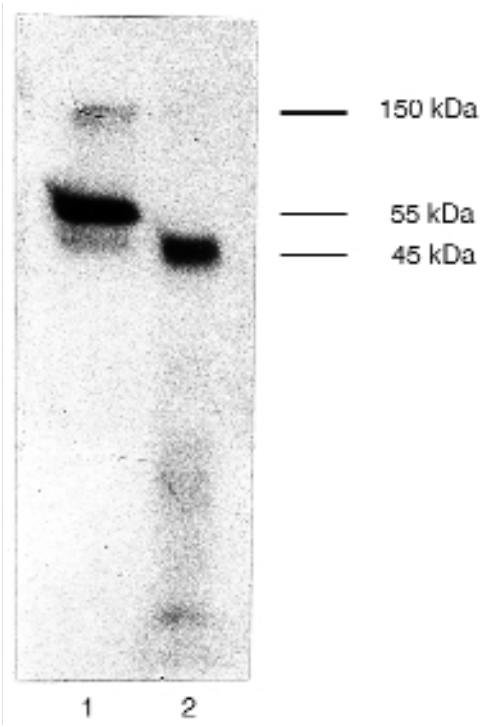

Fig. 1 Autoradiographic analysis of protein phosphorylarion in lung tissue cell extract in the absence (track 1) or presence (track 2) of purified *Legionella* cytolysin. Molecular mass of labeled components is indicated on the right in kilodaltons (kDa). The reaction has been carried out with ^{32}P-γATP. Addition of cytolysin to the reaction mixture resulted in disappearance of 150 kDa and 55 kDa phosphorylated components and presentation of a 45 kDa product. For details see [7]

Our studies, and those of others suggest that cytolysin is important in the pathogenesis of Legionnaires' disease. However, investigations with mutant *Legionella* have shown that *L. pneumophila*, which possesses a specifically inactivated metalloproteinase gene, grows within and kills HL-60 cells, as does the wild-type strain [60]. Similar lethal doses were recorded for guinea pigs challenged with the parental and protease-negative strains, both strains multiplied at similar rates and the histological images of the observed lesions were very similar [17]. It was therefore concluded that the cytotoxic

protease was not required for intracellular infection or multiplication in phagocytes or to kill laboratory animals.

Thus, using a biological approach, several products have been purified from *Legionella* cultures (low-molecular weight cytotoxins and cytolysin). The factors have considerable biological activities and are worthy of further investigation. However, it is clear that the virulence of the bacterium cannot be attributed to these three factors alone. However, attempts to detect other products in *Legionella* directly by their biological activity have failed. This may be because certain bacterial products, if they are not transported into the target cell, even if they are produced in large quantities, have no biological activity and are not detected by biological approaches. Such "hidden" virulence factors may be secreted by legionellae after penetration inside phagocytes thereby crossing the cytoplasmic membrane and gaining direct access to intracellular targets. The infectious agent of legionellosis may be able to "inject" biologically-active products, using specialized delivery apparatus, like that of microorganisms [36].

Biochemical approach Molecular data concerning the pathogenesis of a number of infectious diseases have demonstrated that the biological activity of various virulence factors depends on certain types of enzymatic activity (e.g. ADP-ribosylation, adenylyl cyclase, protein kinase, phospholipase, phosphatase) [8]. Thus, for *Legionella* infection, it may be possible to detect new virulence factors by testing their enzymatic activity using a biochemical approach [14].

At the end of 1979, Baine and colleagues demonstrated that the growth of *L. pneumophila* on agar medium supplemented with hen's egg yolk was accompanied by the development of a cloudy precipitate and iridescence around *Legionella* colonies [5]. This indicated phospholipase activity in *Legionella* cultures. Indeed, using tritiated lecithin, phospholipase C activity has been detected in *L. pneumophila*, *L. bozemanii*, *L. dumoffii*, *L. longbeachae* and *L. jordanis* [4]. In subsequent investigations phospholipase C was purified from *L. pneumophila* strain [3]. The purified protein migrates in SDS-PAGE with an apparent molecular weight of 50–54 kDa. Its activity is maximal at basic pH and can be increased by adding sorbitol, divalent cations and nonionic detergents.

The isolated enzyme has no hemolytic activity, is not toxic to laboratory animals or embryonated hen's eggs. The only insight into possible functions of this product has been obtained with cell culture models using human neutrophils. It has been shown that phospholipase C purified from *L. pneumophila* inhibits several neutrophil functions linked with bactericidal activity [24]. This effect on polymorphonuclear phagocytes may result from the enzymatic degradation of eukaryotic lecithin. Lecithinase may split this phospholipid, which is present in considerable quantities in eukaryotic cells, to produce a potent second messenger (diacylglycerol) thereby affecting eukaryotic signaling reactions that proceed via diacylglycerol protein kinase pathway [8].

Another type of enzymatic activity (protein kinase activity) was detected by Saha and colleagues in crude sonicate of *L. micdadei* using mixed histones as a substrate. Attempts to purify the corresponding enzyme resulted in the isolation of two proteins, both with phosphorylation activity, protein kinase 1 (PK1) and protein kinase 2 (PK2) [54]. The two enzymes have similar pK and optimal pH values. PK2 has been shown to be cAMP- and cGMP-dependent. Its activity is stimulated by calmodulin, Ca^{2+}-calmodulin and Ca^{2+}-phospholipid mixtures. In contrast, PK1 has been shown to be cyclic nucleotide-independent and is not activaded by calmodulin or Ca^{2+} mixtures. PK1 was studied in more detail in further experiments [55].

The homogeneous PK1 was subjected to electrophoresis in SDS-polyacrylamide gels and gave a single band with a molecular weight of 55 kDa. This enzyme catalyzes the phosphorylation of several neutrophil proteins, with molecular weights of 11 to 38 kDa, in both the cytosol and membrane fractions of eukaryotic cells. PK1 modifies brain tubulin and phosphatidylinositol. The phosphatidylinositol-phosphate produced from phosphatidylinositol was not further phosphorylated to give phosphatidylinositol-diphosphate.

Thus, *L. micdadei* contains at least two enzymes that phosphorylate numerous eukaryotic substrates. The physiological substrates for protein kinase 1 include tubulin and phosphatidylinositol. The modification of tubulin may result in defects in chemotaxis, changes to phagosome formation and the failure of phagosome-lysosome fusion. The phosphorylation of phosphatidylinositol may cause abnormalities in various signaling pathways in eukaryotic cells [8]. Another product of *L. micdadei*, acid phosphatase 2 (see below), is able to dephosphorylate phosphatidylinositol-diphosphate to give phosphatidylinositol-phosphate. Thus, the concerted action of protein kinase and phosphatase could sharply increase the level of phosphatidylinositol-phosphate. Another possibility is the formation by *Legionella* protein kinase of an isomeric form of phosphatidylinositol phosphate (e.g. a 3-isomer instead of 4-isomer) with different signaling activity.

Phosphatase activity has been detected in *Legionella* in cytochemical and biochemical investigations in several laboratories. Attempts to purify the corresponding enzyme were made by Saha et al. [53]. In their study, two proteins with phosphatase activity were isolated from bacterial lysates of *L. micdadei*. Acid phosphatase 1 (ACP1) had a molecular weight of approximately 150 kDa and produced no discrete bands on isoelectric focusing gels. Acid phosphatase 2 (ACP2) was approximately 86 kDa in size with a pI = 4.5.

The substrates for ACP2 included phosphothreonine, phosphoserine and phosphotyrosine. The other feature of the purified enzyme was its activity against phosphatidylinositol 4,5-diphosphate and inositol triphosphate [52]. The reaction with phosphatidylinositol 4,5-diphosphate was specific because none of the phosphatidylinositol-4 phosphate formed was further dephosphorylated to phosphatidylinositol. Cell studies using

neutrophils labeled with $^{32}P_i$ have confirmed these results and demonstrated that *Legionella* phosphatase 2 considerably decreases the amount of intracellular phosphatidylinositol-diphosphate, inositol triphosphate and *sn*,-1,2-diacylglycerol.

The activity of *Legionella* phosphatase may affect lipid signaling reactions via at least two possible mechanisms [24]. First, the dephosphorylation of phosphatidylinositol-diphosphate to phosphatidylinositol-phosphate reduces the amount of precursor available to produce inositol triphosphate and diacylglycerol. In addition, inositol triphosphate is further dephosphorylated by the same phosphatase. Second, an increase in the amount of phosphatidylinositol-phosphate alters signal transduction per se because this second messenger has distinct signaling activities [8].

A clear role for acid phosphatase 2 in the intracellular proliferation of legionellae has been demonstrated in human phagocytes. Saha and colleagues, studying the biological activities of purified *L. micdadei* phosphatases, demonstrated strong inhibition of fMLP- and ConA-stimulated O_2^- production after the incubation of human neutrophils with ACP2 [53]. Further work is required to clone the corresponding genes. This should facilitate the subsequent characterization of these factors and confirm their role in the pathogenesis of Legionnaires' disease and intracellular replication of the bacterium.

In our laboratory we have tried, unsucessfully, to detect adenylyl cyclase in *L. pneumophila* cultures. In other experiments using ^{32}P-NAD as a cofactor, we obtained results indicating that ultrasonic lysates of *L. pneumophila* contained an enzyme that modified several bacterial and eukaryotic proteins (Fig. 2). As such a reaction could be ADP-ribosylation, we studied the observed phenomenon more precisely [12, 13]. We purified the putative ADP-ribosyltransferase, and the corresponding gene was cloned and sequenced. Database searches for related proteins revealed strong homology (more than 50% identity at the amino acid level) with NAD-dependent DNA ligases. Purified *Legionella* enzyme was also able to ligate pUC19 DNA cut with restriction endonucleases, at efficiency similar to that of commercial T4 DNA ligase [Belyi et al., in preparation]. Thus, the enzyme that we referred to as "ADP-ribosyltransferase" is in fact a DNA-ligase.

Similar cases have been described. For example, Nozaki and colleagues detected an enzyme in cultures of *Helicobacter pylori* that, in the presence of ^{32}P-NAD performed automodification [41]. Initially the reaction was suspected to be auto-ADP-ribosylation catalyzed by an ADP-ribosyltransferase from *H. pylori*. However, more detailed studies showed that the reaction was the auto-adenylation of an NAD-dependent DNA ligase.

Thus, using a biochemical approach, several important *Legionella* enzymes have been detected in bacterial cultures, purified and investigated. Such an approach is likely to be fruitful in the future as well. However, its successful application depends on thorough knowledge about the enzymatic reactions associated with the virulence of microorganisms in general and about the antibacterial mechanisms of eukaryotic cells. In other words, the search for new activities in *Legionella* cultures requires an understanding of which enzymes in bacteria are important for pathogenesis of diseases in general.

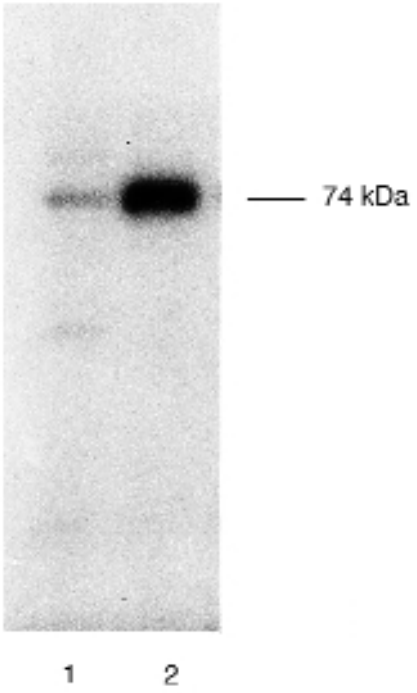

Fig. 2 Autoradiographic analysis of protein modification in the presence of ^{32}P-NAD. The reaction has been carried out with the addition of ultrasonic lysate of *Legionella pneumophila* (track 1) or purified putative "ADP-ribosyltransferase" (track 2) to the reaction mixture. Note automodification of 74 kDa putative *Legionella* "ADP-ribosyltransferase". For details see [13]

Genetic approach One group of researchers has successfully used molecular genetics to study the pathogenesis of *Legionella* infection. Tools were developed to induce and analyze, in cell cultures, mutations in *L. pneumophila* genes that resulted in attenuated phenotypes of the bacterium. The identification of point mutations and the sequencing of the corresponding genes made it possible to identify novel virulence markers in *Legionella*.

Mutations in a gene coding for a 24 kDa surface protein result in a severe reduction in virulence towards macrophages, macrophage-like cell lines, alveolar epithelial cells and protozoa. They also caused considerable attenuation of *L. pneumophila* for laboratory animals [19–22]. As such mutants are impaired in their ability to initiate macrophage infection, the mutated surface component was named Mip (macrophage infectivity potentiator) protein.

The deduced amino acid sequence of the Mip protein from *L. pneumophila* shows its homology to human, *Neurospora* and yeast proteins able to bind the immunosupressant drug FK506 [25]. FK506-binding proteins are receptors belonging

to a family of peptidyl-prolyl *cis/trans* isomerases (PPIs) called immunophilins, which catalyze the *cis/trans* interconversion of prolyl imidic peptide bonds in proteins. Investigations with the 24 kDa Mip protein confirmed that it had isomerase activity. In addition, the inhibitory effect of FK506 on Mip was similar to that on human FK506-binding protein [27].

The structural composition of Mip protein has been addressed in specific studies. Hacker and Fischer suggested that the N-terminus of Mip, which is predicted to be a 60-amino acid α-helix, anchors the protein to the bacterial cell wall [30]. The C-terminus, which carries a domain possessing peptidyl-prolyl *cys/trans* isomerase, projects distally from the bacterial surface and accomplishes its biological function. Additional data from an X-ray solution scattering study suggested that, to perform its function, the Mip protein should be a dimer.

Futher studies showed that *mip*-like genes and their corresponding proteins are present in many strains of *Legionella* as well as in other intracellular microorganisms (*Chlamydia, Coxiella, Rickettsia*) [38, 47a]. Thus, Mip protein has a general and important role in the initiation of a cycle of intracellular parasitism. However, the mechanism of action of Mip protein is unclear.

Recently, a different type of isomerase has been purified and investigated [56]. The enzyme belongs to a family of cyclophilin-like peptidyl-prolyl *cis/trans* isomerases and was named Lcy (*Legionella* cyclophilin-like isomerase). Unlike to FK506-binding proteins, this type of isomerase appears to be widely distributed in various microorganisms, both pathogenic and non-pathogenic for humans. Until recently, cyclophilin-like PPIs have been classed as house-keeping enzymes, participating in general processes of microbial metabolism and being of limited importance in the pathogenesis of infectious diseases. To test whether the cyclophilin-like peptidyl-prolyl *cis/trans* isomerase of *L. pneumophila* is involved in intracellular parasitism, an Lcy-negative strain was generated and tested in a cell culture model with *A. castellanii*. The mutated strain was one tenth as invasive as wild-type legionellae. Thus, in addition to Mip protein, another PPI seems to be involved in the virulence of *Legionella*, promoting the intracellular multiplication of the infectious agent by an unidentified mechanism.

In other studies, several loci of the *Legionella* chromosome encoding proteins necessary for intracellular multiplication of the bacterium have been identified and characterized. Shuman and colleagues detected two well separated chromosome fragments that carried genes important for intracellular parasitism in *L. pneumophila* and which were therefore called *icm* (intracellular multiplication genes). One such locus contained a set of 18 genes [18, 43, 51, 57]. Mutation in 16 of these genes resulted in the production of strains incapable of killing macrophages. Study of the organization of this locus revealed that *icm* genes are organized into four operons and several independent genes. Most of the Icm proteins are apparently located on the bacterial inner or outer membrane or in the periplasm. Most Icm proteins have no significant homology to known proteins in database. However, four products (IcmE, IcmL, IcmO and IcmP) shared sequence similarity to plasmid genes required for conjugation. Genes of another region, *icmWXYZ*, are also required for the growth of *L. pneumophila* in macrophages. Only *icmW* was found to have weak sequence similarity to conjugation proteins.

Due to these sequence similarities, attempts have been made to study the effect of the mutational inactivation of *icm* genes on conjugation efficiency in *L. pneumophila*. In these investigations, *icmT* and *icmR* mutants had conjugation efficiencies less than one thousandth that of the wild type and mutations in *icmF*, *icmE* and *icmC* gave conjugation frequencies one tenth to one hundredth that of the wild type. Inactivation of other genes of this locus (including *icmL*, *icmO* and *icmP* with considerable similarity of the corresponding proteins to conjugation proteins) had no effect on conjugation.

Isberg and colleagues used a different strategy to identify products of *L. pneumophila* involved in the intracellular proliferation of the bacterium. The authors generated a panel of transposon-induced attenuated mutants and located genes that restored the ability of microorganisms to produce defects in organelle trafficking and the formation of replicative phagosomes in macrophages. Such genes were therefore named *dot* (defect in organelle trafficking).

The first gene detected, *dotA*, was found to be adjacent to the *icmWXYZ* region. It encodes a large product with no apparent homology to any known protein in the database. DotA was predicted to be an inner membrane protein with eight membrane-spanning domains [50]. DotA-negative mutants reside in a phagosome with no apparent inhibition of its membrane maturation. Mutant strains do not prevent phagosome-lysosome fusion and do not recruit organelles to produce replicative vacuoles [49]. Thus, unlike the parental strain, DotA$^-$ mutants were conveyed by the normal endocytic pathway through phagosome-lysosome fusion to killing.

Other *dot* genes necessary for the intracellular proliferation of *L. pneumophila* have been detected [63]. They are organized into two regions. One region contains three *dot* genes located approximately 10 kb from *icmWXYZ*. Another region consists of 11 genes. As for Icm proteins, several *dot* products have some sequence similarity to proteins of the bacterial conjugation system.

Abu Kwaik and colleagues identified 32 transposon-induced mutants of *L. pneumophila* with defects in intracellular survival and replication within macrophages. Surprisingly, most of these mutants (26 strains) had a wild-type phenotype during the infection of protozoa and only 6 mutants had mild defects in proliferation in *Acanthamoeba polyphaga*. Based on this observation, the corresponding genes were called *mil* (macrophage-specific infectivity loci) [29].

Thus, the molecular genetic approach has identified a large number of genes encoding proteins important for the intracellular proliferation of *L. pneumophila*. They include the

Table 1 Putative virulence determinants of *Legionella* (for details see corresponding chapters of the review).

Detected by biological approach	Detected by biochemical approach	Detected by molecular-genetic approach
Low molecular mass cytotoxin	Phospholipase C	Peptidyl-prolyl cis/trans isomerases
Low molecular mass lethal toxin	Protein kinases	Loci *icm*/*dot*
Cytolysin	Phosphatases	Loci *mil*

gene for Mip protein, involved in the initiation of macrophage infection, *icm* genes, necessary for the proliferation of *L. pneumophila* within and the killing of macrophages, *dot* genes, necessary for specific organelle recruitment, changes in phagosome maturation and the inhibition of phagosome-lysosome fusion in macrophages, and *mil* genes, the products of which are important for *Legionella* infection of macrophages but not of protozoa.

The product of *mip* has been well studied both from biochemical and molecular-genetic points of view. However, its mechanism of action is unknown. Genes coding for Icm, Dot and Mil proteins are now being intensively studied. Their interrelationship is not clear. It appears (at least for *icm* and *dot*) that many of the loci described independently by different groups are identical. This has already been confirmed for *icmO* and *icmP*, which have been shown to be homologous to *dotL* and *dotM*.

The molecular mechanism of action of Icm-Dot proteins is unknown. It is generally believed, however, that the products of *icm-dot* genes build a large supramolecular complex for the transportation of effector molecules of *L. pneumophila* into eukaryotic cells. Similar structures have been described for several intracellular parasites (e.g. *Salmonella* and *Shigella*) and are referred to as the "Type III secretion system" [36]. Using such transportation systems, these microorganisms "inject" certain biologically-active products directly into eukaryotic cells, providing them with access to intracellular targets. The ability of *L. pneumophila* to lyse erythrocytes and to have a cytotoxic effect on human macrophages in a contact-dependent manner is consistent with this idea [35]. It is strange, however, that none of the described Icm-Dot proteins has any homology with known components of type III secretion systems in *Salmonella*, *Shigella* or *Yersinia*. It is therefore possible that *Legionella* has a primarily different type of translocation machinery.

Conclusion

Bacteria of the genus *Legionella* are typical intracellular parasites. They invade and proliferate in different cells, including both "professional" and "non-professional" phagocytes. Molecular mechanisms of host cell-*Legionella* interactions are only beginning to be elucidated. Using biological, biochemical and molecular genetics approaches, a panel of bacterial products has been detected and characterized (Table 1). However, their interactions during cellular invasion by bacteria are unknown.

The most impressive data have been obtained recently with the detection of the *icm*, *dot* and *mil* genes. However, the proteins they encode have little sequence homology to known products. Therefore, the determination of their molecular mechanism of action and their involvement in virulence are complicated. Attempts to detect new enzymatic activities in *Legionella* cultures could therefore be fruitful and important for understanding mechanisms of intracellular parasitism. Such enzymes may be effector components of the presumed Icm-Dot secretion apparatus. However, one should bear in mind that the production of such enzymes may be under strict genetic and environmental control and the mechanisms of regulation of *Legionella* virulence are completely unknown.

Thus, despite intensive research activity, many aspects of the interaction of *Legionella* with its host cell are unclear. Future studies using different approaches are needed to clarify the molecular mechanisms of virulence in *Legionella*, a major human pathogen and a fascinating model microorganism for investigations into intracellular parasitism.

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